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The inhibitory effects of rebamipide on cigarette smoke-induced airway mucin production ☆

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Summary Cigarette smoke may be the main cause of chronic bronchitis. Exposure of cigarette smoke induces the recruitment of inflammatory cells in the airway epithelium, and release of the tumor necrosis factor α (TNFα) from airways. Previous reports have shown that cigarette smoke induces goblet cell metaplasia by activating an epidermal growth factor receptor (EGFR) cascade, and that this results in mucin production. Rebamipide (2-(4-chlorobenzoylamino)-3-[2(1H)-quinolinon-4-yl] propionic acid, OPC-12759) directly inhibits the production of superoxide (O_2^-) and inhibits proinflammatory cytokines (such as TNFα and IL-8). In the present study, we aimed to analyze the inhibitory effects of rebamipide on TNFα and EGFR activation after cigarette smoke treatment in vitro and in vivo.

NCl-H292 cells and Sprague-Dawley rats were used for in vitro and in vivo studies. In vitro studies, cigarette smoke solution was found to increase TNFα secretion, and EGFR-specific tyrosine phosphorylation, and to elevate MUC5AC production. These effects were inhibited dose-dependently by pretreatment with rebamipide (MUC5AC protein levels were inhibited from 44% to 17%, $P<0.05$). In vivo studies, cigarette smoke was found to cause inflammatory cell recruitment and to increase the secretion of TNFα in bronchoalveolar lavage (BAL) fluids (from 198 ± 78 to 2270 ± 158 pg/ml, $P<0.01$). Moreover, the pretreatment of rats with rebamipide inhibited goblet cell metaplasia and TNFα secretion, dose-dependently (from 2270 ± 158 to 1377 ± 112 pg/ml, $P<0.05$).

In conclusion, the exposure of airway epithelium to cigarette smoke-induced TNFα production, neutrophil recruitment, activated EGFR, and caused MUC5AC

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mucin synthesis. Moreover, rebamipide was found to prevent this cigarette smoke-induced TNFα release, and mucin production.

Introduction

The hallmarks of chronic bronchitis are cough and excessive mucus production, and cigarette smoke is viewed as a major etiologic factor of chronic bronchitis. Exposure to cigarette smoke has been reported to induce goblet cell metaplasia and mucus production, the recruitment of inflammatory cells, including neutrophils, in the airway epithelium, and the release of tumor necrosis factor α (TNFα) from airway epithelium.[1,2] Mucin MUC5AC is a major mucin in the airway epithelium.[3] Mucin synthesis in airways has been reported to be regulated by the epidermal growth factor receptor (EGFR) system.[4–8] Moreover, EGFR is known to be upregulated by the proinflammatory cytokine TNFα, which upregulated in hypersecretory airway diseases.[5–9] Moreover, stimulation with TNFα induces EGFR expression in human epithelial cells in vitro and in rats in vivo, and the activation of EGFR by its ligands or by oxidative stress results in mucin production.[4,6,7,10] Activated neutrophils release oxygen-free radicals, which cause mucin synthesis via EGFR transactivation, and these effects are blocked by EGFR tyrosine kinase inhibitors and also by antioxidants. In addition, cigarette smoke induces goblet cell metaplasia by activating an EGFR cascade which releases TNFα, resulting in mucin expression.[11]

Rebamipide (2-(4-chlorobenzoylamino)-3-[2(1H)-quinolinon-4-yl] propionic acid, OPC-12759) is a gastro-protective agent used in the treatment of gastritis and ulcerative colitis; however, the mechanisms of its anti-inflammatory action are not completely understood.[12–14] Nevertheless, rebamipide is known to directly inhibit the production of superoxide (O_2^-) and to eliminate reactive oxygen species (ROS) in hydroxyl radical (OH) system.[15–18] In addition, rebamipide protects the gastric mucosa against acute injury caused by various noxious and ulcerogenic factors. Recently, several reports have shown that rebamipide works as an anti-inflammatory agent in both acute and chronic inflammation, and has an inhibitory effect on proinflammatory cytokines (such as TNFα and IL-8) and on the activation of neutrophils.[19–21]

In the present study, we investigated the inhibitory effects of rebamipide on cigarette smoke-induced mucin production in airway epithelium, since the compound is known as inhibitor of proinflammatory cytokines, neutrophil activation, and oxidative stress.

Materials and methods

In vitro studies

Preparation of cigarette smoke solution: Standard research cigarettes (code 2R4F, produced by the Tobacco and Health Research Institute, University of Kentucky) were used. Cigarette smoke solution was prepared as previously described.[11] In brief, cigarette smoke was withdrawn into a polypropylene syringe (35 ml) at a rate of one puff/min and then bubbled slowly into 20 ml of RPMI 1640 medium containing 50 mM HEPES buffer. The smoke solution was then titrated to pH 7.4 and used immediately after preparation.

Cell culture: NCI-H292 cells, a human pulmonary mucoepidermoid carcinoma cell line, were grown in RPMI 1640 medium containing 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and HEPES (25 mM) at 37 °C in a humidified 5% CO_2 water-jacketed incubator. Cells were cultured in either six-well culture plates or eight-chamber slides. When confluent, cells were incubated for 1 h in cigarettes smoke solution. They were then washed and incubated with fresh medium alone. Experiments were terminated at 24 h for protein measurements. As controls, NCI-H292 cells were incubated with medium alone in an identical manner. In inhibition studies, NCI-H292 cells were pretreated with rebamipide (10, 30 μg/ml; generously provided by Otsuka Pharma, Korea), with the EGFR tyrosine kinase inhibitor, AG1478 (10 μM; Calbiochem, San Diego, CA, USA), or with TNFα monoclonal antibody, infliximab (3, 6, 9, 12 μg/ml) 30 min before they were incubated in cigarette smoke solution. Infliximab was purchased from a pharmaceutic supplier (Schering Korea Ltd., Seoul, Korea).

Immunoblotting for activated EGFR: Cells were serum starved for 24 h and then stimulated with cigarette smoke solution for 15 min. After stimulation, cells were lysed with lysis buffer (20 mM sodium phosphate, pH 7.8, 150 mM NaCl, 5 mM EDTA, 50 mM HEPES, 1% Triton X-100, 50 mM NaF, 1 mM sodium orthovanadate, 5 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of leupeptin

and aprotinin) and incubated for 30 min at 4 °C. To remove insoluble materials, cell lysates were centrifuged at 14,000 rpm for 5 min at 4 °C. Aliquots of supernatants containing equal amounts of protein were suspended in SDS sample buffer and boiled for 5 min. Proteins were separated by SDS-PAGE in 8% acrylamide gels. The resulting gels were equilibrated in transfer buffer: 25 mM Tris–HCl, 192 mM glycine, and 20% (vol/vol) methanol, pH 8.3, and proteins were transferred electrophoretically to nitrocellulose membranes. The membranes were incubated with 5% fat-free skimmed milk in phosphate-buffered saline (PBS) containing 0.05% Tween 20 for 1 h and then incubated with anti-phospho-specific EGFR monoclonal antibody (EGFR-p; 1:1000; 1 μg/ml; Calbiochem, San Diego, CA, USA) and anti-EGFR (Ab-2) monoclonal antibody (EGFR; 1:1000; 100 μg/ml; Oncogene, La Jolla, CA, USA) overnight. Bound antibodies were visualized according to a standard protocol for the avidin–biotin–alkaline phosphatase complex method (ABC kit; Vector Laboratories, Burlingame, CA, USA).

Measurement of TNFα: NCI-H292 cells were incubated with various concentrations of cigarette smoke solution or were treated in culture medium with or without various concentrations of rebamipide (10 μg/ml or 30 μg/ml). TNFα levels were determined in the supernatants harvested after 6, 12, 24 and 48 h by ELISA (Sigma, St Louis, MO, USA).

Immunoassay of MUC5AC protein: NCI-H292 cells were incubated with various concentrations of cigarette smoke solution or were treated in culture medium with rebamipide, AG1478 or TNFα monoclonal antibody (infliximab). MUC5AC protein levels were measured as described previously.[7] In brief, cell lysates were prepared with PBS at multiple dilutions, and 50 μl of each sample was then incubated with bicarbonate–carbonate buffer (50 μl) at 40 °C in a 96-well plate (Maxisorp Nunc; Fisher Scientific, Santa Clara, CA, USA) until dry. The plates were then rewashed three times with PBS and blocked with 2% bovine serum albumin, fraction V (Sigma, St Louis, MO, USA) for 1 h at room temperature. The plates were then rewashed three times with PBS and incubated with 50 μl of MUC5AC monoclonal antibodies (clone 45 M1, 1:100; Neo-Markers, Fremont, CA, USA) diluted with PBS containing 0.05% Tween 20. After 1 h, the wells were washed three times with PBS, and 100 μl of horseradish peroxidase-goat anti-mouse IgG conjugate (1:10,000) was dispensed into each well. After a further 1 h, the plates were washed three times with PBS. Color reaction was developed using 3,3′,5,5′-tetramethylbenzidine peroxidase solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) and stopped with 2 N H_2SO_4. Absorbance was read at 450 nm. Each experiment was repeated at least five times. Mucin of the submandibular gland was used as a positive ELISA control and naïve medium (RPMI+10% FBS, never exposed to cells) was used a negative control. The interassay variation was less than 5%.

In vivo studies

Induction of goblet cell metaplasia by cigarette smoke exposure: Male Sprague-Dawley rats weighing 150 g were used for the study. Animals were housed in a temperature- and humidity-controlled room and had free access to water and standard laboratory food. Animals were assigned at random to the nonsmoking control group or to the smoke-exposed control or treatment groups. Five animals were studied in each group. Rats in the smoking groups were exposed to five cigarettes a day for 5 days (code 2R4F, produced by the Tobacco and Health Research Institute, University of Kentucky). On each day of exposure, animals were placed individually inside a Plexiglas cabinet (40 × 90 × 100 mm). Cigarette smoke was delivered into the cabinet by air inflow at a rate of 1.7 ml/s through a burning cigarette in the chamber. The combustion time of the cigarette was ∼3 min. A ventilator inside the cabinet ensured rapid and equal distribution of smoke. Fresh air was delivered into the cabinet to remove the smoke. Animals were exposed to cigarettes at intervals of 30 min. During exposure, animals, including control animals, did not receive food or water, but were allowed free access to both after exposure.

Inhibition of cigarette smoke-induced goblet cell metaplasia by rebamipide: To evaluate the effect of rebamipide on goblet cell metaplasia and mucus production, animals were treated once daily with vehicle (distilled water) or with rebamipide in vehicle at doses of 300 or 1000 mg/kg orally 1 day before exposure to cigarette smoke. Animals were euthanized 10 days after exposure to cigarette smoke.

Bronchoalveolar lavage (BAL) cell and cytokine analysis: Animals were euthanized 1 day after the last exposure to cigarette smoke for BAL. BAL fluid was used for cytospin preparations. The slides were fixed and stained with Diff-Quick (Baxter Healthcare, McGaw Park, IL, USA), and differential cell counts were obtained by using light microscopic evaluation of 300 cells/slide. Total BAL cell counts were performed with a hemocytometer. After removing cells from BAL fluid by centrifugation, 100-μl aliquots were analyzed for the presence of TNFα by ELISA, using commercially available kits by

following the manufacturer's instructions (Genzyme, Cambridge, MA, USA).

Tissue preparation: Animals were euthanized with a lethal dose of pentobarbital sodium (200 mg/kg, intraperitoneal) 5 days after the session of cigarette smoke, and the systemic circulation was perfused with 1% paraformaldehyde in diethylpyrocarbonate (Sigma, St Louis, MO, USA)-treated PBS via the left ventricle. For paraffin sections, tissues were placed in 4% paraformaldehyde overnight, dehydrated with ethanol, and embedded in paraffin. The embedded tissues were cut as cross sections 4 μm thick and placed on glass slides.

Quantification of goblet cell metaplasia: In all studies, the carina was examined to obtain consistent sampling. We measured Alcian blue (AB)–periodic acid-Schiff (PAS)-positive areas and total epithelial area, and we expressed the results as the percentages of AB–PAS stained areas to total epithelial area. Stained slides were examined under a light microscope (Olympus BX51, Tokyo, Japan) connected to a video camera (Olympus DP 50, Tokyo, Japan) and images were captured using software (Viewfinder Lite v 1.0, Pixera Co, Los gatos, CA, USA). Airway epithelium images were recorded in six consecutive high-power fields at $\times 400$. Images were analyzed using the SigmaScan Pro program (SPSS INC, Chicago, IL, USA).

Statistics

All data are expressed as means ± standard error of mean (SE). One-way analysis of variance (ANOVA) was used to determine statistically significant differences between groups. Scheffé's *F*-test was used to correct for multiple comparisons when statistical significances were identified in the ANOVA. $P < 0.05$ for the null hypothesis was accepted as indicating a statistically significant difference.

Results

In vitro studies in NCI-H292 cells

Cigarette smoke induced EGFR tyrosine phosphorylation: Because the activation of EGFR leads to MUC5AC synthesis,[7] we examined the effect of cigarette smoke solution on activation of the EGFR tyrosine kinase. Immunoblot analysis of cell lysates identified that cigarette smoke solution induced EGFR-specific tyrosine phosphorylation. This effect was inhibited by pretreating NCI-H292 cells with AG1478 or with rebamipide. EGFR expression was not significantly different for the different lysates, thus indicating equal protein loading (Fig. 1).

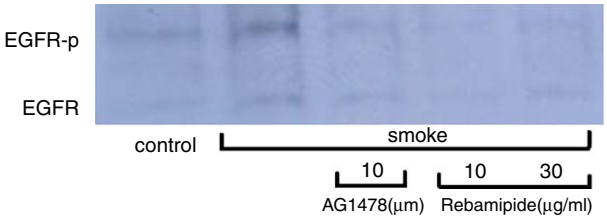

Figure 1 Tyrosine phosphorylation of EGFR induced by cigarette smoke was inhibited by AG1478 and rebamipide. NCI-H292 cells were incubated under the conditions shown for 15 min and lysed. Proteins were separated by SDS-PAGE in 8% acrylamide gels and blotted with anti-phospho-specific EGFR monoclonal antibody and anti-EGFR (Ab-2) monoclonal antibody. Cigarette smoke increased EGFR-specific tyrosine phosphorylation in NCI-H292 cells. The effect was inhibited by AG1478 and rebamipide (bottom, which showed even loading of total protein in each lane).

Rebamipide inhibited cigarette smoke-induced TNFα production by NCI-H292 cells

TNFα can induce EGFR expression, which results in MUC5AC production.[7] Therefore, we measured TNFα levels in cigarette smoke-stimulated NCI-H292 cells at 6, 12, 24, and 48 h. Peak TNFα levels were observed at 24 h (not shown). NCI-H292 cells were treated with various doses of cigarette smoke and TNFα levels were measured in the culture supernatants of the cells. Cigarette smoke solution induced dose-dependently TNFα production from NCI-H292 cells (not shown), whereas TNFα production did not increase in cells pretreated with rebamipide. Thus, rebamipide had an inhibitory effect on TNFα production in cigarette smoke-stimulated NCI-H292 cells, dose-dependently (Fig. 2).

Rebamipide prevents MUC5AC production in NCI-H292 cells: NCI-H292 cells were treated with various doses of cigarette smoke. Cigarette smoke solution caused increase MUC5AC protein synthesis dose-dependently, and this effect was prevented by pretreatment of the cells with rebamipide, dose-dependently (Fig. 3). Increased MUC5AC protein synthesis was near completely inhibited by AG1478. Also we examined whether cigarette smoke solution induced MUC5AC protein synthesis was influenced by TNFα. Cigarette smoke solution induced MUC5AC protein synthesis was inhibited by TNFα monoclonal antibody (infliximab), dose-dependently (Fig. 4).

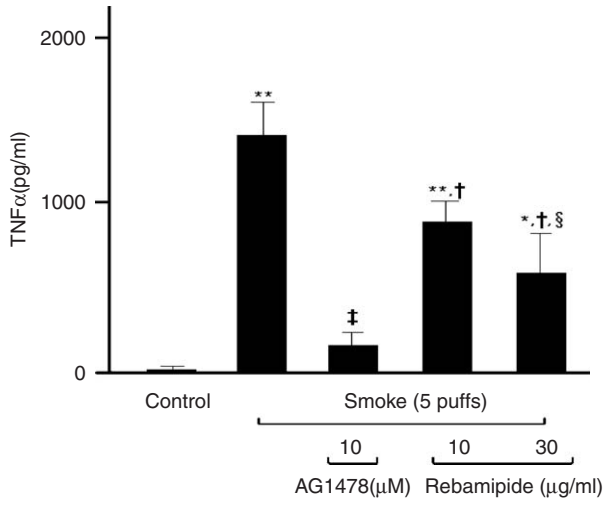

Figure 2 Cigarette smoke-induced TNFα secretion was inhibited by rebamipide dose-dependently in NCI-H292 cells ($n = 5$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control, $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ compared with five puffs of cigarette smoke solution alone, $^{\S}P < 0.05$ compared with rebamipide 10 μg/ml).

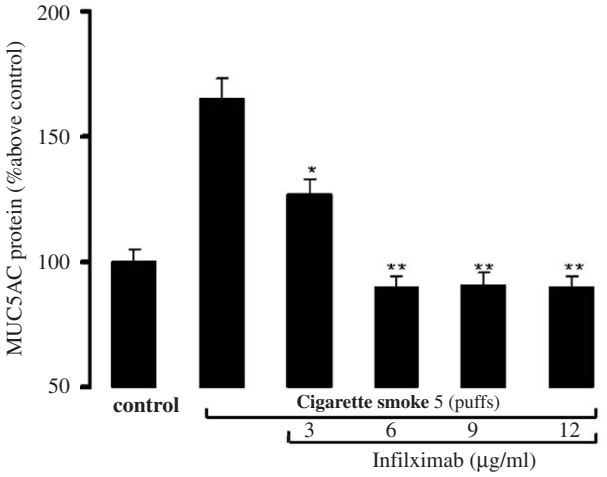

Figure 4 Effects of TNFα inhibitor (infliximab) on smoking solution induced MUC5AC production in NCI-H292 cells. Cigarette smoke solution induced MUC5AC protein synthesis was inhibited by TNFα monoclonal antibody (infliximab), dose-dependently. ($n = 5$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with five puffs of cigarette smoke solution alone). MUC5AC protein levels were measured by ELISA.

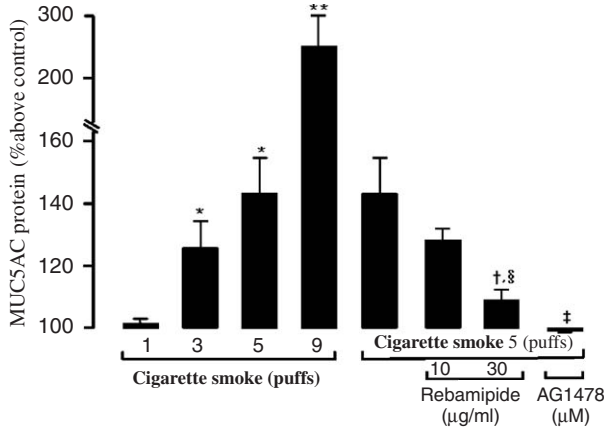

Figure 3 Effects of cigarette smoke solution and rebamipide on MUC5AC production in NCI-H292 cells. Cigarette smoke solution increased MUC5AC production dose-dependently ($n = 5$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control, $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ compared with five puffs of cigarette smoke solution alone, $^{\S}P < 0.05$ compared with rebamipide 10 μg/ml), and this increase was inhibited dose-dependently by rebamipide. MUC5AC protein levels were measured by ELISA.

In vivo studies in rats airways

Cigarette smoke-stimulated rats induces airway inflammation and TNFα production: Because activated neutrophils have been implicated in mucin production in airway epithelial cells and because neutrophils are the predominant leukocytes recruited into airways by cigarette smoke, we examined by using BAL fluid analysis. The BAL fluids of control rats contained few inflammatory cells, but the stimulation of rat airways with cigarette smoke caused inflammatory cell recruitment, predominantly neutrophils and macrophages. Moreover, pretreatment with rebamipide inhibited the recruitment of neutrophils in BAL fluids (Fig. 5A).

TNFα has been shown to induce EGFR expression in airway epithelium, and thus, we examined the effect of cigarette smoke on TNFα secretion in BAL fluids. In control rats, TNFα secretion was minimal. However, stimulation with cigarette smoke increased TNFα secretion in BAL fluids of cigarette smoking group (Fig. 5B), and rebamipide inhibited this cigarette smoke-induced TNFα secretion.

Cigarette smoke increases goblet cell production in rat airways: In control animals, the airway epithelium was stained sparsely by AB/PAS. The inhalation of cigarette smoke (five cigarettes/day for 5 days) resulted in a marked increased in AB–PAS stained areas (Fig. 6A). Moreover, pretreatment of rats with rebamipide inhibited goblet cell metaplasia dose-dependently (Fig. 6B). These results indicate that rebamipide prevents cigarette smoke-induced mucin production in rat airways.

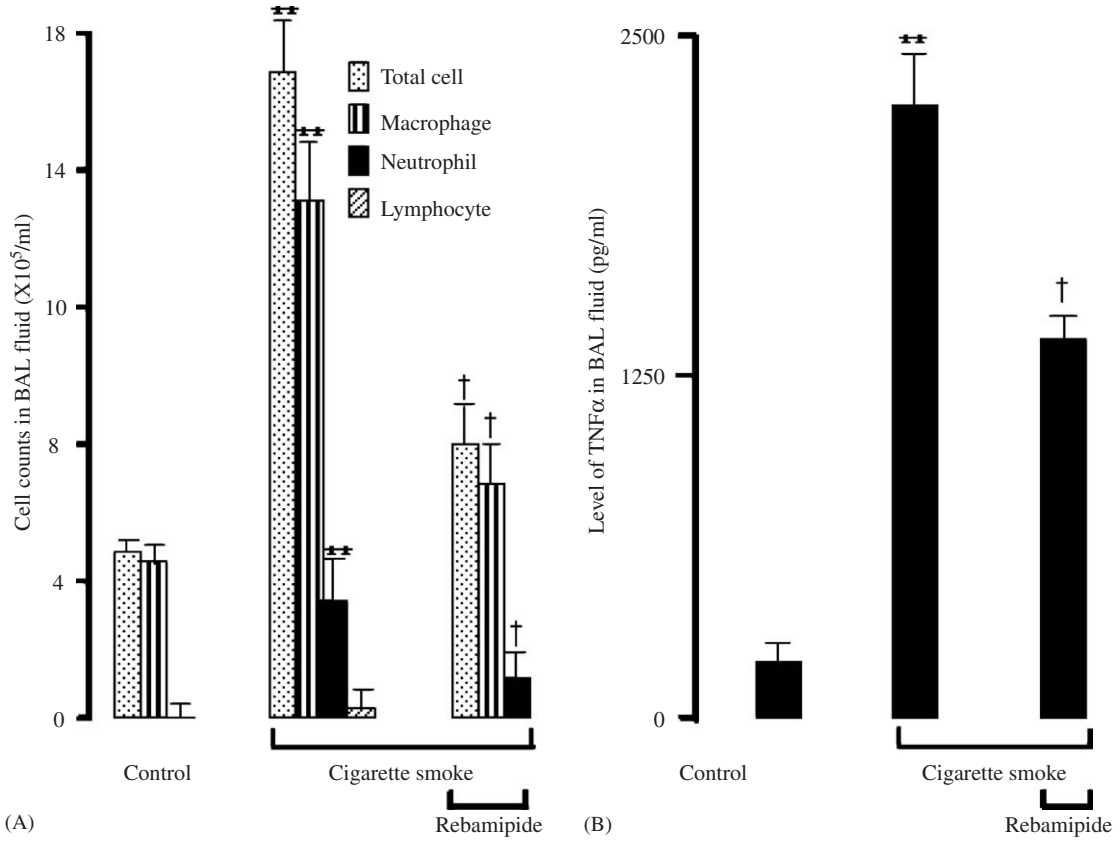

Figure 5 Effects of cigarette smoke on leukocyte recruitment (A) and on TNFα secretion (B) in BAL fluids. Cigarette smoke increased neutrophil recruitment. Pretreatment with rebamipide (300 mg/kg) inhibited cigarette smoke-induced leukocyte recruitment ($n = 5$, $^{**}P < 0.01$ compared with control, $^{\dagger}P < 0.05$ compared with cigarette smoke alone). ELISA analysis showed that cigarette smoke significantly increased in TNFα ($n = 5$; $^{**}P < 0.01$ compared with control). Rebamipide inhibited cigarette smoke-induced TNFα secretion ($^{\dagger}P < 0.05$ compared with cigarette smoke alone).

Discussion

In the present study, we investigated whether rebamipide prevents mucus production in the cigarette smoke-stimulated airway epithelium, because rebamipide has an inhibitory effect on TNFα production and recruitment of neutrophils on airway. Our results show that exposure of the airway epithelium to cigarette smoke induces the release of TNFα and the recruitment of neutrophils, which activate EGFR, and MUC5AC mucin synthesis. Pretreatment with rebamipide prevents this release of TNFα from airway epithelium and the recruitment of neutrophils into the airway epithelium, indicating that rebamipide prevents cigarette smoke-induced MUC5AC mucin production.

The main risk factor of COPD is cigarette smoking, which is closely associated with mucus hypersecretion. Exposure to cigarette smoke activates an inflammatory cascade in the airway epithelium, which results in the production of a number of potent cytokines and chemokines, damage to the lung epithelium, increased mucus secretion, and the recruitment of macrophages and neutrophils into the airway epithelium.[22] Multiple mechanisms have been suggested to be responsible for cigarette smoke-induced mucus hypersecretion. One of the reported mechanisms involves EGFR upregulation, and EGFR tyrosine kinase activation by its ligand or its transactivation by oxidative stress, thus causing mucin MUC5AC expression at both the mRNA and protein levels in airway epithelial cells in vitro, or mucin MU5AC production and goblet cell metaplasia in vivo.[11] EGFR activation may involve two pathways, namely, ligand-dependent and ligand-independent EGFR tyrosine phosphorylation.[23] In ligand-dependent EGFR tyrosine phosphorylation, EGFR ligands (EGF or TGFα) bind to EGF receptors in the extracellular domain and activate them while, in ligand-independent EGFR tyrosine phosphorylation, EGFR tyrosine phosphorylation occurs in the absence of exogenous EGFR ligands. According to Shao et al., metalloprotease TNFα-converting enzyme (TACE) is

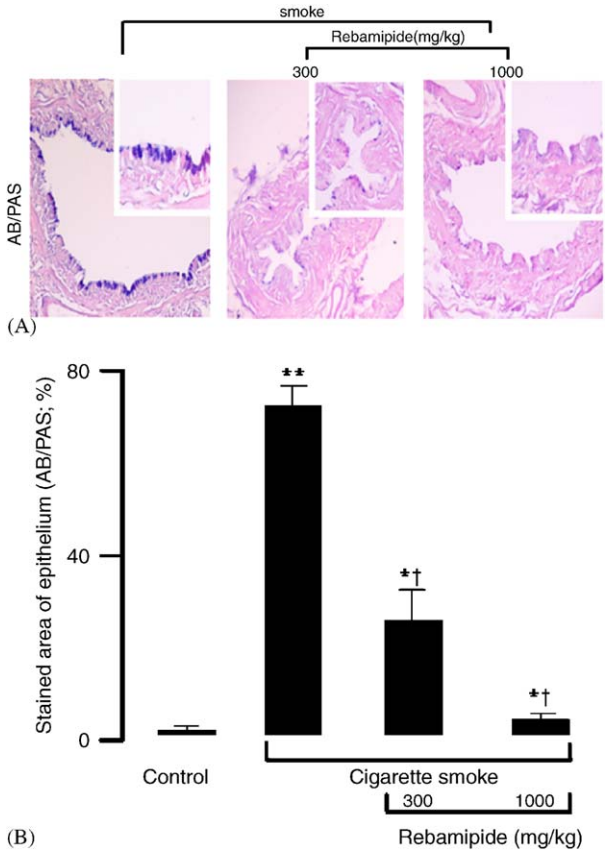

Figure 6 Photomicrographs of rat airway epithelium stained with AB/PAS(A) and the % area stained by AB/PAS in airway epithelium (B). Cigarette smoke increased AB/PAS staining. Pretreatment with rebamipide (300 and 1000 mg/kg day orally) prevented the cigarette smoke-induced effects. Results are representative of those for five rats ($^{*}P<0.05$, $^{**}P<0.01$ compared with control, $^{\dagger}P<0.05$, $^{\ddagger}P<0.01$ compared with five puffs of cigarette smoke alone). Photomicrographs are shown at $\times 20$ magnification; *insets*, $\times 40$ magnification.

activated by cigarette smoke, resulting in cleaving EGFR proligand (TGFα which is constitutively expressed in airway epithelial cells), leading to EGFR phosphorylation and mucin induction.[24,34] Ligand-independent EGFR phosphorylation is reported in response to oxidative stress that can be produced by cigarette smoke and by activated neutrophils.[10,11,25] Cigarette smoke inhalation increased TNFα, oxygen-free radical production and neutrophil recruitment in airways. Ultimately, cigarette smoke inhalation activated EGFR and increased mucin MUC5AC production by proinflammatory cytokines and neutrophils.

TNFα is a potent proinflammatory cytokine and is elevated in the sputum of patients with COPD.[26] Although the mechanisms by which TNFα causes cytokine stimulation and apoptosis have been well studied, comparatively little is known about the mechanisms by which TNF stimulates EGFR. In recent years, evidence has emerged that implicates TNFα in the regulation of mucus secretion. In particular, TNFα has been shown to induce mucus hypersecretion in human airway epithelial cells in culture.[27,28] Moreover, the mucin produced by TNFα was found to be mediated by MUC5AC or MUC2 gene expression.[29,30] The transactivation of EGFR by TNFα has been previously described.[31–33] Chen et al.[32] demonstrated the role of EGFR signaling in TNF-induced cell proliferation using a selective pharmacological inhibitor of EGFR kinase activity.

Rebamipide is an anti-ulcer drug, and has been reported to prevent various acute experimental gastric lesions and to accelerate chronic gastric ulcer healing.[12,35] In particular, rebamipide has known cytoprotective effects in humans and animals.[36] For example, rebamipide was found to enhance gastric mucosal defense by increasing the COX-2-dependent production of PGs in gastric mucosa.[37] In addition, rebamipide increased mucin release in an eye model of inflammation (the *N*-acetylcysteine model). However, it is not known whether changes in mucus expression in the *N*-acetylcysteine model accurately reflect what occurs during normal aqueous tearing or during aqueous tear deficiency.[38] Recent studies have also reported that rebamipide has a beneficial effect on inflammatory diseases; rebamipide is known as inhibitor of proinflammatory cytokines, neutrophil activation, and oxidative stress.[20,39,40] However, the mechanisms underlying these effects are not well understood.

In our study, cigarette smoke was found to induce significant TNFα release and MUC5AC protein synthesis from NCI-H292 cells (Figs. 2 and 3), and rebamipide was found to inhibit TNFα release dose-dependently, and to inhibit cigarette smoke-induced MUC5AC production. And this increased MUC5AC protein synthesis was inhibited by TNFα monoclonal antibody (Fig. 4). Together these findings indicate that TNFα is a regulator of MUC5AC protein synthesis.

In vivo studies, little TNFα protein expression was observed in control animals, but animals exposed to cigarette smoke showed TNFα and neutrophils upregulation, which could have resulted in goblet cell metaplasia (Figs. 5 and 6). Thus, pretreatment with rebamipide was found to prevent TNFα release and neutrophil recruitment in cigarette smoke-stimulated airways, and to inhibit goblet cell metaplasia. These results imply that rebamipide treatment could reduce mucin production in cigarette smoking model.

In conclusion, we found that the exposure of the airway epithelium to cigarette smoke induces neutrophil recruitment, TNFα release, and upregulates EGFR expression, causing MUC5AC mucin synthesis. Rebamipide was found to prevent TNFα release, neutrophil recruitment into the airways, and MUC5AC mucin synthesis in cigarette smoke-stimulated airway epithelium. These results suggest that rebamipide may be used to treat mucus hypersecretion in cigarette smokers.

References

1. Nadel JA. Role of neutrophil elastase in hypersecretion during COPD exacerbations, and proposed therapies. *Chest* 2000;**117**(5 Suppl 2):386S–9S.
2. Puljic R, Pahl A. Smoke induced changes in epithelial cell gene expression: development of an in vitro model for COPD. *Altex* 2004;**21**(1):3–7.
3. Dohrman A, Miyata S, Gallup M, Li JD, Chapelin C, Coste A, et al. Mucin gene (MUC 2 and MUC 5AC) upregulation by Gram-positive and Gram-negative bacteria. *Biochim Biophys Acta* 1998;**1406**(3):251–9.
4. Nadel JA. Mechanisms of airway hypersecretion and novel therapy. *Chest* 2000;**117**(5 Suppl 1):262S–6S.
5. Nadel JA, Burgel PR. The role of epidermal growth factor in mucus production. *Curr Opin Pharmacol* 2001;**1**(3):254–8.
6. Shim JJ, Dabbagh K, Ueki IF, Dao-Pick T, Burgel PR, Takeyama K, et al. IL-13 induces mucin production by stimulating epidermal growth factor receptors and by activating neutrophils. *Am J Physiol Lung Cell Mol Physiol* 2001;**280**(1):L134–40.
7. Takeyama K, Dabbagh K, Lee HM, Agusti C, Lausier JA, Ueki IF, et al. Epidermal growth factor system regulates mucin production in airways. *Proc Natl Acad Sci USA* 1999;**96**(6): 3081–6.
8. Takeyama K, Fahy JV, Nadel JA. Relationship of epidermal growth factor receptors to goblet cell production in human bronchi. *Am J Respir Crit Care Med* 2001;**163**(2):511–6.
9. Lee HM, Takeyama K, Dabbagh K, Lausier JA, Ueki IF, Nadel JA. Agarose plug instillation causes goblet cell metaplasia by activating EGF receptors in rat airways. *Am J Physiol Lung Cell Mol Physiol* 2000;**278**(1):L185–92.
10. Takeyama K, Dabbagh K, Jeong Shim J, Dao-Pick T, Ueki IF, Nadel JA. Oxidative stress causes mucin synthesis via transactivation of epidermal growth factor receptor: role of neutrophils. *J Immunol* 2000;**164**(3):1546–52.
11. Takeyama K, Jung B, Shim JJ, Burgel PR, Dao-Pick T, Ueki IF, et al. Activation of epidermal growth factor receptors is responsible for mucin synthesis induced by cigarette smoke. *Am J Physiol Lung Cell Mol Physiol* 2001;**280**(1):L165–72.
12. Yamasaki K, Kanbe T, Chijiwa T, Ishiyama H, Morita S. Gastric mucosal protection by OPC-12759, a novel antiulcer compound, in the rat. *Eur J Pharmacol* 1987;**142**(1):23–9.
13. Joh T, Takezono Y, Oshima T, Sasaki M, Seno K, Yokoyama Y, et al. The protective effect of rebamipide on paracellular permeability of rat gastric epithelial cells. *Aliment Pharmacol Ther* 2003;**18**(Suppl 1):133–8.
14. Ogino K, Hobara T, Ishiyama H, Yamasaki K, Kobayashi H, Izumi Y, et al. Antiulcer mechanism of action of rebamipide, a novel antiulcer compound, on diethyldithiocarbamate-induced antral gastric ulcers in rats. *Eur J Pharmacol* 1992;**212**(1):9–13.
15. Kojima M, Iwakiri R, Wu B, Fujise T, Watanabe K, Lin T, et al. Effects of antioxidative agents on apoptosis induced by ischaemia-reperfusion in rat intestinal mucosa. *Aliment Pharmacol Ther* 2003;**18**(Suppl 1):139–45.
16. Shimoyama T, Fukuda S, Liu Q, Fukuda Y, Nakaji S, Sugawara K. Characteristics of attenuating effects of rebamipide, an anti-ulcer agent, on oxidative burst of human neutrophils. *J Pharmacol Sci* 2003;**91**(2):153–7.
17. Seo JY, Kim H, Seo JT, Kim KH. Oxidative stress induced cytokine production in isolated rat pancreatic acinar cells: effects of small-molecule antioxidants. *Pharmacology* 2002; **64**(2):63–70.
18. Sakurai K, Yamasaki K. Protective effect of rebamipide against hydrogen peroxide-induced hemorrhagic mucosal lesions in rat stomach. *Jpn J Pharmacol* 1994;**64**(4): 229–34.
19. Masamune A, Yoshida M, Sakai Y, Shimosegawa T. Rebamipide inhibits ceramide-induced interleukin-8 production in Kato III human gastric cancer cells. *J Pharmacol Exp Ther* 2001; **298**(2):485–92.
20. Aihara M, Imagawa K, Funakoshi Y, Ohmoto Y, Kikuchi M. Effects of rebamipide on production of several cytokines by human peripheral blood mononuclear cells. *Dig Dis Sci* 1998;**43**(9 Suppl):160S–6S.
21. Hong KW, Kim KE, Rhim BY, Lee WS, Kim CD. Effect of rebamipide on liver damage and increased tumor necrosis factor in a rat model of endotoxin shock. *Dig Dis Sci* 1998;**43**(9 Suppl):154S–9S.
22. Adler KB, Fischer BM, Wright DT, Cohn LA, Becker S. Interactions between respiratory epithelial cells and cytokines: relationships to lung inflammation. *Ann N Y Acad Sci* 1994;**725**:128–45.
23. Burgel PR, Nadel JA. Roles of epidermal growth factor receptor activation in epithelial cell repair and mucin production in airway epithelium. *Thorax* 2004;**59**(11):992–6.
24. Shao MX, Nakanaga T, Nadel JA. Cigarette smoke induces MUC5AC mucin overproduction via tumor necrosis factor-{alpha} converting enzyme in human airway epithelial (NCI-H292) cells. *Am J Physiol Lung Cell Mol Physiol* 2004:L420–7.
25. Goldkorn T, Balaban N, Matsukuma K, Chea V, Gould R, Last J, et al. EGF-Receptor phosphorylation and signaling are targeted by H_2O_2 redox stress. *Am J Respir Cell Mol Biol* 1998;**19**(5):786–98.
26. Vernooy JH, Kucukaycan M, Jacobs JA, Chavannes NH, Buurman WA, Dentener MA, et al. Local and systemic inflammation in patients with chronic obstructive pulmonary disease: soluble tumor necrosis factor receptors are increased in sputum. *Am J Respir Crit Care Med* 2002; **166**(9):1218–24.
27. Fischer BM, Krunkosky TM, Wright DT, Dolan-O'Keefe M, Adler KB. Tumor necrosis factor-alpha (TNF-alpha) stimulates mucin secretion and gene expression in airway epithelium in vitro. *Chest* 1995;**107**(3 Suppl):133S–5S.
28. Levine SJ, Larivee P, Logun C, Angus CW, Ognibene FP, Shelhamer JH. Tumor necrosis factor-alpha induces mucin hypersecretion and MUC-2 gene expression by human airway epithelial cells. *Am J Respir Cell Mol Biol* 1995;**12**(2): 196–204.
29. Song KS, Lee WJ, Chung KC, Koo JS, Yang EJ, Choi JY, et al. Interleukin-1 beta and tumor necrosis factor-alpha induce MUC5AC overexpression through a mechanism involving ERK/p38 mitogen-activated protein kinases-MSK1-CREB activation in human airway epithelial cells. *J Biol Chem* 2003; **278**(26):23243–50.
30. Lin J, Haruta A, Kawano H, Ho SB, Adams GL, Juhn SK, et al. Induction of mucin gene expression in middle ear of rats by

tumor necrosis factor-alpha: potential cause for mucoid otitis media. *J Infect Dis* 2000;**182**(3):882–7.
31. Chobotova K, Muchmore ME, Carver J, Yoo HJ, Manek S, Gullick WJ, et al. The mitogenic potential of heparin-binding epidermal growth factor in the human endometrium is mediated by the epidermal growth factor receptor and is modulated by tumor necrosis factor-alpha. *J Clin Endocrinol Metab* 2002;**87**(12):5769–77.
32. Chen WN, Woodbury RL, Kathmann LE, Opresko LK, Zangar RC, Wiley HS, et al. Induced autocrine signaling through the epidermal growth factor receptor contributes to the response of mammary epithelial cells to tumor necrosis factor alpha. *J Biol Chem* 2004;**279**(18):18488–96.
33. Palombella VJ, Yamashiro DJ, Maxfield FR, Decker SJ, Vilcek J. Tumor necrosis factor increases the number of epidermal growth factor receptors on human fibroblasts. *J Biol Chem* 1987;**262**(5):1950–4.
34. Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, et al. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature* 1997;**385**(6618):729–33.
35. Yamasaki K, Ishiyama H, Imaizumi T, Kanbe T, Yabuuchi Y. Effect of OPC-12759, a novel antiulcer agent, on chronic and acute experimental gastric ulcer, and gastric secretion in rats. *Jpn J Pharmacol* 1989;**49**(4):441–8.
36. Kawano S, Sato N, Kamada T, Yamasaki K, Imaizumi T, Komemushi S. Protective effect of rebamipide (OPC-12759) on the gastric mucosa in rats and humans. *Nippon Yakurigaku Zasshi* 1991;**97**(6):371–80.
37. Sun WH, Tsuji S, Tsujii M, Gunawan ES, Kawai N, Kimura A, et al. Induction of cyclooxygenase-2 in rat gastric mucosa by rebamipide, a mucoprotective agent. *J Pharmacol Exp Ther* 2000;**295**(2):447–52.
38. Urashima H, Okamoto T, Takeji Y, Shinohara H, Fujisawa S. Rebamipide increases the amount of mucin-like substances on the conjunctiva and cornea in the N-acetylcysteine-treated in vivo model. *Cornea* 2004; **23**(6):613–9.
39. Murakami K, Okajima K, Harada N, Isobe H, Okabe H. Rebamipide prevents indomethacin-induced gastric mucosal lesion formation by inhibiting activation of neutrophils in rats. *Dig Dis Sci* 1998;**43**(9 Suppl):139S–42S.
40. Iinuma S, Naito Y, Yoshikawa T, Takahashi S, Takemura T, Yoshida N, et al. In vitro studies indicating antioxidative properties of rebamipide. *Dig Dis Sci* 1998;**43**(9 Suppl): 35S–9S.